Bioaugmentation for Reduction of Diffuse Pesticide Contamination

A Bioprophylactic Concept

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Abstract

Pesticides and their residues frequently contaminate surface waters and groundwater so consequently there is a great need to identify methods and practices that reduce such contamination. This thesis examined the potential of a 'bioprophylactic' concept based on the hypothesis that diffuse contamination after application in the field can be significantly reduced if pesticides are degraded as rapidly as possible after the intended effect has been achieved. This involves adding pesticide degraders together with the pesticide at the time of application. Such enhanced degradation is particularly important for pesticides used on non-agricultural soils with low degradation and high leaching potential (*e.g.* roadsides, railway embankments, paths, farmyards, urban land). The herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA) was used as a model herbicide in this thesis, together with its degrader *Sphingobium* sp. T51. Soil inoculation with *Sphingobium* sp. T51 in microcosms significantly enhanced the degradation of MCPA compared with an uninoculated soil, without losing the herbicidal effect. In sand, 10^5 cells g^{-1} dry weight of soil reduced the MCPA concentration by 79-86% within one day, while only 0-31% reduction was observed in the uninoculated control after three days. However, *Sphingobium* sp. T51 needs to be formulated to retain its viability and degradation activity during prolonged storage and also for protection from the harsh environmental stresses associated with its application and functioning in the field. Both freeze drying and fluidised bed drying of *Sphingobium* sp. T51 resulted in high initial cell survival rates, of 67-85%. The storage stability of formulated *Sphingobium* sp. T51 was dependent on the formulation excipient/carrier used, storage temperature and atmospheric conditions. *Sphingobium* sp. T51 demonstrated approximately 50% survival in a freeze-dried sucrose formulation after six months of storage under partial vacuum at 25 °C. A fluidised bed-dried formulation with cottonseed flour as carrier resulted in the highest MCPA degradation in microcosms, but the storage stability was insufficient. Formulated and long-term stored *Sphingobium* sp. T51 reduced pesticide leaching by 85-94% of the total amount added in sand columns. Future research needs regarding practical implementation of the concept are discussed.

Keywords: bioaugmentation, pesticide degradation, formulation, leaching, diffuse contamination, *Sphingobium*, MCPA

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Till min familj

Fantastiskt är att höra barnskratt och ha jord under naglarna.

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Cederlund, H., Börjesson, E., Önneby, K. & Stenström, J (2007). Metabolic and cometabolic degradation of herbicides in the fine material of railway ballast. *Soil Biology & Biochemistry* 39(2), 473-484.

II Önneby K., Jonsson A. & Stenström J. (2010). A new concept for reduction of diffuse contamination by simultaneous application of pesticide and pesticide-degrading microorganisms. *Biodegradation* 21 (1), 21-29.

III Önneby K., Pizzul L., Bjerketorp J., Mahlin D., Håkansson S. & Wessman P (2013). Effects of di- and polysaccharide formulations and storage conditions on survival of freeze dried *Sphingobium* sp. *World Journal of Microbiology and Biotechnology* DOI: 10.1007/s11274-013-1303-7. In press.

IV Önneby K., Håkansson S., Pizzul L. & Stenström J. Reduced leaching of the herbicide MCPA after bioaugmentation with a formulated and stored *Sphingobium sp.* Submitted.

Papers I – III are reproduced with the permission of the publishers.

The contribution of Karin Önneby to the papers included in this thesis was as follows:

I Performed the laboratory work dealing with MCPA and analysis of MCPA. Made a minor contribution to writing the manuscript.

II Planned the work together with the co-authors. Performed the laboratory work and data analysis. Wrote the manuscript together with Stenström.

III Planned the work together with the co-authors. Performed the laboratory work and wrote the manuscript together with the co-authors.

IV Planned the work together with the co-authors. Performed the laboratory work. Had the main responsibility for writing the manuscript.

Abbreviations

2,4D	2,4-dichlorophenoxyacetic acid
2,4-DB	2,4-dichlorophenoxybutonic acid
2,4-DP	2,4-dichlorophenoxyproponic acid
CA	casamino acids
CFU	colony forming units
DDT	dichlorodiphenyltrichloroethane
Diuron	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
HEC	hydroxyethylcellulose
HPLC	high-performance liquid chromatography
HR-MAS-NMR	high resolution magic angle spinning nuclear magnetic resonance
MCP	4-chloro-2-methylphenol
MCPA	4-chloro-2-methylphenoxyacetic acid
MCPB	4-chloro-2-methylphenoxybutonic acid
MCPP	4-chloro-2-methylphenoxypropionic acid
MS	minimal salt
ROS	radical oxygen species
tfdA-tfdF	genes encoding degradation pathway of phenoxy acids
TSB	tryptic soy broth

1 Introduction

Pesticides are used to control weeds, insect infestations and diseases in both agricultural and non-agricultural applications. However, pesticides and their residues can be transported to other environmental compartments and consequently are frequently detected in groundwater and surface waters (Hildebrandt *et al.*, 2008; Schipper *et al.*, 2008; Maloschik *et al.*, 2007; Törnquist *et al.*, 2007; Gilliom *et al.*, 2006). Although the concentrations detected in waters are typically low (Graaf, 2011; Törnquist *et al.*, 2007; Kreuger & Törnqvist, 1998), they can still exceed the European drinking water limit for pesticides (EU, 2013), which is currently 0.1 µg L^{-1} for a single pesticide and 0.5 µg L^{-1} for the sum of pesticides. In 1999, with an addition in 2005, Sweden adopted environmental quality objectives in 16 areas, of which at least two have a direct connection to pesticide use, *i.e*. "A non-toxic environment" and "Good water quality". In these objectives, two sub-goals are that*:* "*the future use of pesticides shall be sustainable in the long-term at an acceptable risk-level*" and "*residue concentrations of pesticides in surface- and ground waters shall be close to zero*" (Swedish Government Bill, 2013a, 2013b). To date, measures including information campaigns (Kreuger, 1998), improved spraying equipment (Nuyttens *et al.*, 2007), better routines for filling and cleaning sprayer tanks (Castillo *et al.*, 2008) and better application procedures (Arvidsson *et al.*, 2011) have been taken to minimise pesticide losses to the environment. However, to achieve the environmental quality objectives more has to be done. The development of new innovative concepts is necessary to obtain sustainable and environmentally friendly pesticide use.

The aim of the work described in this thesis was to present and investigate a new concept where bioaugmentation (*i.e*. removal of pollutants by inoculation of microorganisms) is used to reduce pesticide contamination from diffuse sources after application in the field. The basic aim of this 'bioprophylactic'

concept is to create optimal conditions for microbial degradation of pesticides before pesticide losses to other environmental compartments can occur. The work focused on non-agricultural uses of pesticides (*e.g.* herbicide use on railways), where the need to find methods for environmental sustainable use of pesticides is especially pressing.

The thesis begins by providing a general background to pesticides and environmental problems related to their use and then focuses on pesticide use on railways (**I**). It continues with a presentation of the bioprophylactic concept (**II**). The need for formulation of microorganisms is then discussed, focusing on storage stability (**III**, **IV**) and maintained biological activity (**IV**), followed by degradation efficiency and leaching in bioaugmented soil columns (**IV**). Finally, the challenges with in-field application of microorganisms are addressed. The herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA), its degrader *Sphingobium* sp. T51 and the railway track as the application site are used as models here, but the same questions and challenges apply to other systems, with other pesticides and degraders, where enhanced pesticide degradation is needed.

2 Pesticides and their environmental fate

2.1 Pesticide use

Pesticides are defined in the Swedish Environmental Code as chemical or biological products intended to prevent or deter animals, plants or microorganisms from causing damage or detriment to human health or damage to property (Swedish Environmental Code, 2013). Pesticides are used in agriculture, horticulture, forestry, transport (railways), industry, parks and households (gardens). Based on the target organism, pesticides can be broadly categorised as:

- Herbicides (for weed control)
- Insecticides (for insect control)
- Fungicides (for fungal pathogen control)
- Others (such as nematicides, bactericides)

Chemical pesticides are not a modern invention, but during the 1940s, when agriculture was industrialised and the insecticide DDT (dichlorodiphenyltrichloroethane) was introduced onto the market, they became more extensively used. DDT was followed by other chlorinated hydrocarbon compounds, phenoxyacetic acids and organophosphorus pesticides. At that time, these products were valued for their persistence in the environment. Over the years the development of new pesticides has been towards more readily biodegradable and also more water-soluble substances that are often less broad-ranging in their effects and more targeted towards pests, weeds or diseases (Rathore, 2012; Troyer, 2001). In 2011, more than 8500 tonnes of pesticide active ingredients (a.i.) were sold in Sweden, of which the majority (>70%) were sold to industry, primarily for wood treatment. The

agricultural sector represented 21% of total sales and 65% of herbicides sold were used on arable soil. The most commonly used herbicides in Sweden in 2011 were glyphosate and MCPA, with 708 and 313 tonnes, respectively (KEMI, 2013a).

Although the immediate benefits of pesticides were obvious, it gradually became apparent that many of these products also had severe drawbacks, affecting nature, animals and people in ways that had not been anticipated. Knowledge about the importance of proper handling was poor in the beginning and it was only much later that the fate of pesticides after use in the field was studied in greater depth, openly discussed and became a topic of public concern. National environmental monitoring of pesticides has been carried out in Sweden by the Swedish University of Agricultural Sciences (SLU) for almost 30 years (Graaf, 2011; Adielsson, 2009; Törnquist *et al.*, 2007). The monitoring data show that the frequency of samples with a total pesticide concentration above 0.5 μg L^{-1} has decreased over the years, both in surface waters and groundwater. These decreasing levels of pesticides have been attributed to increased awareness among farmers of the need for better routines and for correct handling of spraying equipment and application procedures (Nilsson & Kreuger, 2001; Kreuger, 1998). For example, in one area investigated, information campaigns resulted in a reduction in pesticide concentrations of >90% in nearby surface waters (Törnquist *et al.*, 2007). However, the success in reducing pesticide losses to surrounding waters depends on the origin of the contamination.

2.2 Environmental fate of pesticides

2.2.1 Origin of pesticide contamination

The pesticides found in water can originate from point or diffuse sources. Point source contamination refers to pesticide losses after improper handling at the farm, such as from spills during filling and cleaning of sprayer tanks. One effective and relatively simple method to reduce contamination from such sources is the use of biobeds (Torstensson & Castillo, 1997). The composition of the biobed is intended to promote microbial degradation activities and pesticide binding and the idea is that all handling of pesticides should be done on top of the biobed, in which fast degradation and strong pesticide binding give a low risk of leaching of possible spills. This method has been successfully implemented among farmers in Sweden, with more than 1500 biobeds in use today, and the concept has also spread to other countries (Castillo *et al.*, 2008).

Pesticide contamination from diffuse sources is more complicated to deal with than that from point sources. Diffuse source losses result from events or emissions that have no single identifiable point of origin. They occur after application in the field, where the pesticides are exposed to various environmental conditions and are subject to different processes, such as degradation, adsorption to soil colloids and off-site transport.

2.2.2 Off-site transport of pesticides

Pesticide transport can occur by several different routes, *e.g.* volatilisation, surface transport and leaching through the soil profile (Carter, 2000), with pesticides ending up where they can cause damage. Several factors affect how a pesticide will move in the environment once it is introduced. Pesticides that are highly water soluble are often found in surrounding waters in time periods closely related to their use (Kreuger, 1998). Some pesticides are volatile and easily transported through the air. In addition, degradation or transformation of a pesticide results in structural changes that affect how degradation products move in the environment (Flury, 1996). Soil texture (*i.e.* relative percentage of sand, silt and clay) and structure play a large role in the transport processes of pesticides. Coarse-textured soils allow water to percolate faster, giving low sorption of pesticides, and generally do not contain a large microbial population relative to other soil types (Cederlund *et al.*, 2008). Soils that are high in clays and organic matter slow down the movement of water, have a higher sorption capacity and often both a higher biomass and functional diversity of soil organisms that can degrade the pesticide (Vinther *et al.*, 2008; Bending *et al.*, 2002). However, clay soils may result in pesticide losses through preferential flow in soil macropores (Flury, 1996; Bergström & Jarvis, 1993). Pesticides can be transported either dissolved in the soil solution or adsorbed on solid particles (*i.e.* mineral particles or organic matter) (Bergström *et al.*, 2011; Borggaard & Gimsing, 2008; Vereecken, 2005). In addition, factors such as amount of pesticide applied and time and method of application and climate factors such as temperature, wind speed, humidity and rain affect possible routes of pesticide transport in the environment.

2.2.3 Microbial pesticide degradation

Pesticide degradation is often a microbial process and the ability of soil microorganisms to degrade pesticides depends largely on their bioavailability. Bioavailability is generally defined as the quantity of a chemical that is accessible to an organism, and the time-dependent reduction in bioavailability is called ageing (Alexander, 2000). This decrease in bioavailability is

controlled by sorption (Bending & Rodriguez-Cruz, 2007; Spliid *et al.*, 2006; Jensen *et al.*, 2004; Johannesen *et al.*, 2003; Steinberg *et al.*, 1987), together with entrapment in organic matter or diffusion into micropores, which makes it impossible for degrading microorganisms and enzymes to reach the pesticide (Bergström & Stenström, 1998). Consequently, reduced bioavailability leads to increased pesticide persistence and thereby also an increased risk of long-term losses to surface waters and groundwater.

Environmental conditions can greatly affect pesticide biodegradation and soil water content is probably one of the major factors (Cederlund *et al.*, 2012; Cederlund *et al.*, 2008; Schroll *et al.*, 2006). Bacteria in general require high moisture for metabolic activities, although some are more tolerant to drought. Studies have shown that increasing the soil moisture content up to a certain amount usually leads to enhanced degradation rates of pesticides (Grundmann *et al.*, 2011; El Sebai *et al.*, 2010; Taylor-Lovell *et al.*, 2002). Diffusion of the pesticide to the degrader is required for degradation and low soil moisture contents could therefore be a limiting factor regulating pesticide bioavailability and degradation (Bosma *et al.*, 1997). Indeed, degradation of pesticides under water saturation can be substantial, as such conditions facilitate transport (Lauga *et al.*, 2013). Pesticide degradation in very dry conditions can also occur, although with low degradation rates (**IV**; Chatterjee *et al.*, 2013). Sandy soils, with their low water-holding capacity (Ritchie, 1981), limit microbial activity in general and thus also pesticide degradation (**IV**; Cederlund *et al.*, 2012; Alletto *et al.*, 2006), but much of the water present is available to microorganisms. In contrast, the water in soils with a high clay content is often strongly bound (Gong *et al.*, 2003) and thus not available for microorganisms.

Soil temperature influences the microbial composition of the soil and each microbial species has a definite range of temperature preference for its activity. Thus, the composition of active microbial species varies in soils as the temperature changes. However, higher temperatures do not automatically give larger microbial populations and activities (Jensen *et al.*, 2003). Certain microorganisms are well adapted to colder climates and efficient degradation can occur even at rather low temperatures (Welander, 2005).

There is often a negative correlation between soil pH and pesticide degradation rates (**I**; Grundmann *et al.*, 2007; Walker *et al.*, 2001). Bacteria often have a very narrow pH interval with neutral or slightly alkaline conditions favoured (Rousk *et al.*, 2009; Bending *et al.*, 2003). Low soil pH can affect the activity of the pesticide-degrading microorganisms directly, but can also decrease microbial degradation indirectly due to lower water solubility of the pesticide (Flury, 1996).

Soil is generally considered to be substrate-deficient and the availability of essential nutrients such as carbon, nitrogen, phosphorus and oxygen can also limit the pesticide degradation rate (Alexander, 1999). Addition of substrate generally gives a rapid increase in total microbial activity (Stenström *et al.*, 2001), but such an increase may not necessarily result in increased pesticide degradation, as the latter also depends on pesticide availability and the presence of microorganisms capable of pesticide degradation.

2.2.4 Degradation kinetics

Microorganisms that can utilise a pesticide as a substrate for growth will increase in numbers and when the conditions are optimal this can result in exponential growth (Bergström & Stenström, 1998) (Figure 1a). Such growth-linked degradation has been found for example for 2,4-dichlorophenoxyacetic acid (2,4-D) (Stenström, 1989; Kunc & Rybarova, 1983), MCPA (**I**; Gozdereliler *et al.*, 2013), atrazine (Yanzekontchou & Gschwind, 1994) and 3-chlorobenzoate (Qi *et al.*, 2007) and for several other pesticides (Sørensen & Aamand, 2003; Robertson & Alexander, 1994; Torstensson *et al.*, 1975). The biological efficiency of converting the pesticide into biomass (*i.e*. the yield) varies considerably (Robertson & Alexander, 1994; Stott *et al.*, 1983), and depends on the species, the substrate and its concentration, environmental conditions and the presence of other organisms (Stott *et al.*, 1983).

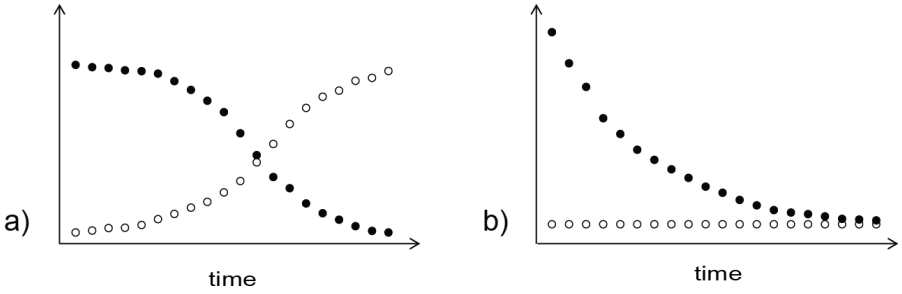

Figure 1. a) Growth-linked (metabolic) and b) non-growth-linked (co-metabolic) pesticide degradation. Filled circles are pesticide concentration and open circles cell numbers.

Pesticides can also be degraded co-metabolically (Figure 1b). Co-metabolism is defined as the transformation of a non-growth substrate (co-substrate) by microorganisms that grow on a primary substrate (Horvath *et al.*, 1990). Many pesticides are subject to co-metabolic degradation, including glyphosate (Torstensson & Aamisepp, 1977), isoproturon (Bending *et al.*, 2001), carbofuran (Robertson & Alexander, 1994) and diuron (**I;** Sørensen *et al.*, 2003). Degradation by co-metabolism often follows first-order kinetics

(Torstensson & Aamisepp, 1977) and does not support growth of the active microorganisms, and therefore no acclimatisation occurs after repeated applications of the pesticide (Gimsing *et al.*, 2004). Instead, the degradation rate is often related to some measure of the general microbial activity of the soil (Ghafoor *et al.*, 2011).

2.3 Pesticide contamination from non-agricultural uses

Arable soil can contain billions of microorganisms per gram of soil and pesticide degradation is often not a problem but, as described above, pesticide losses can still occur under certain conditions. In contrast, for many non-agricultural uses of pesticides, *e.g.* on roadsides, paths, farmyards and in cities (Spliid *et al.*, 2004), the conditions for degradation are poor due to both a low density and low activity of microorganisms. In addition, these soils generally have a low sorption capacity, resulting in high potential for leaching of pesticides. The soil along railway tracks is a typical example of such 'problem' soils.

2.3.1 Weed control on railways

Herbicides are applied regularly on railways for weed control. Although the consumption in Sweden is small (2.92 tonnes a.i. year^{-1}; mean 2002-2007, H. Cederlund, pers. comm. 2013), corresponding to approximately 0.14% of the total pesticide use in Sweden (KEMI 2013a), environmentally friendly use of herbicides on railways and in areas with similar soils is a difficult challenge. The railway embankment is made up of stones, gravel and sand to a suitable level and above this the tracks are placed in a layer of macadam or gravel (approximately 20-30 cm). This construction contains very low amounts of organic matter and fine material, such as clay, to allow maximum drainage (Torstensson *et al.*, 2005), leading to concerns that herbicide use can result in water contamination. Over the years, different herbicides have been tested for use on Swedish railways. Atrazine, diuron and imazapyr are examples of herbicides that have been detected in groundwater pipes during such tests (Börjesson *et al.*, 2004; Ramwell *et al.*, 2004; Torstensson, 2001). Currently, only glyphosate is approved for weed control on railways in Sweden. However, glyphosate has no effect on some weeds, such as the very frequent common horsetail (*Equisetum arvense*) (Torstensson *et al.*, 2005). Therefore, research efforts are underway to find new herbicides that give acceptable weed control and that are tolerable from an environmental point of view.

2.3.2 Microbial degradation of pesticides in the ballast soil

Both the microbial biomass and activity are low in railway embankments (Cederlund & Stenström, 2004). Compared with arable or forest soils, the microbial biomass is several orders of magnitude lower in railway embankments (Cederlund *et al.*, 2008), leading to herbicide degradation also being slow (Börjesson *et al.*, 2004; Torstensson *et al.*, 2002). On the other hand, the availability of the herbicide for microbial degradation is high in the coarse-textured soil. Therefore, pesticide degradation of herbicides that can be used as substrate for growth would possibly lead to substantially increased degradation rates, as the degradation would not be limited by the initial amount of degraders present. In Paper **I**, microbial degradation of the herbicides diuron and MCPA, which possess different degradation kinetics, was studied in the fine soil of railway embankments. MCPA is a candidate for use on Swedish railways due to its acceptable herbicidal effect on, for example, common horsetail (H. Cederlund, pers. comm. 2013) and its generally rapid growth-linked degradation in arable soil (Sørensen *et al.*, 2006; Smejkal *et al.*, 2001c; Tett *et al.*, 1997; Hoffmann *et al.*, 1996). Diuron is one of the most widely used herbicides for weed control on railway tracks worldwide, but was prohibited for use in Sweden in 1993. It is suggested to be co-metabolically degraded (Sørensen *et al.*, 2003) and has been shown to be highly persistent in ballast soils and also susceptible to leaching (Skark *et al.*, 2004; Torstensson *et al.*, 2002).

Indeed, Paper **I** shows that the degradation of diuron followed first-order kinetics, thus indicating co-metabolic degradation. The slow degradation, with half-life values ranging between 122-365 days, was correlated to the microbial biomass (measured as substrate induced respiration, SIR) and reflected the overall low microbial activity in the ballast soil (**I**). It possibly also indicated that bacteria capable of rapid diuron degradation were not present (Sørensen *et al.*, 2001). This was further supported by Cederlund *et al*. (2008), who found that many potentially ecologically relevant functional groups are not present, or at least not active, in ballast soil.

Degradation of MCPA typically followed growth-linked degradation and was relatively rapid compared with that of diuron (**I**). In ballast soil with no history of MCPA use, the number of MCPA degraders was below the limit of detection (<20 cells per g dw of soil). The number of MCPA degraders was higher in soil previously treated with MCPA, although with high variability, indicating that MCPA degraders are unevenly distributed throughout the railway embankment. In addition, yield estimates of MCPA degraders were correlated to the nitrogen content, indicating that MCPA degradation is

nitrogen-limited in ballast soil (**I**). It has also been shown that the microbial activity is correlated with soil organic matter content (measured as loss of ignition) in ballast soil, suggesting that the microorganisms are located in hot-spots rich in organic material (Cederlund *et al.*, 2008; Cederlund & Stenström, 2004).

The laboratory results presented in Paper **I** demonstrate the important principal difference between different degradation kinetics. In this case, the fast degradation rate of the metabolically degraded MCPA would appear to make it a more suitable choice for weed control on railways, at least in comparison with the non-growth supporting diuron. However, the uneven distribution of degraders in the railway embankment makes the overall degradation difficult to predict. In addition, MCPA is known for its high mobility in soils and has been detected in groundwater pipes in concentrations above the EU limit (*i.e.* 0.1 µg L^{-1} water) after being applied on railways (Table 1). It is therefore not permitted for use on Swedish railways.

Table 1. *Concentration of MCPA (µg L^{-1}) in groundwater pipes on the railway at Vikstadalsbanan after application of 1.5 L ha^{-1} MCPA750. A, B and C represent different sections of the track (H. Cederlund, pers. comm. 2013)*

Time after application (days)	***MCPA (µg L^{-1})***		
	Section A	***Section B***	***Section C***
-4	0.08	0.05	<0.05
3	0.12	0.17	<0.05
9	5.57	0.46	0.9
17	0.78	0.25	0.09
92	<0.05	<0.05	<0.05

3 The bioprophylactic concept

3.1 A new concept for enhanced pesticide degradation

Enhanced pesticide degradation is clearly needed on sites such as railway embankments. As the bioavailability decreases with time, the concept of enhanced pesticide degradation is based on the idea that pesticides should be degraded as fast as possible after the intended effect has been obtained and before ageing and transport processes occur. For this to happen, sufficient numbers of highly efficient and evenly distributed pesticide degraders are needed. As previously described, this is generally not the natural case in railway embankments (**I**) or comparable soils. One way to increase the number of active degraders could be to add, together with the pesticide, microorganisms that can degrade the pesticide and thus overcome catabolic limitations and distribution of indigenous microorganisms in pesticide degradation (**II**) (Figure 2). The basic hypothesis behind the bioprophylactic concept is that microorganisms which can degrade many of the pesticides used today can be enriched and isolated from natural materials (soil, plants, waters *etc*.), cultivated and formulated in a way appropriate for the application, and finally inoculated at places where enhanced degradation of pesticides is needed.

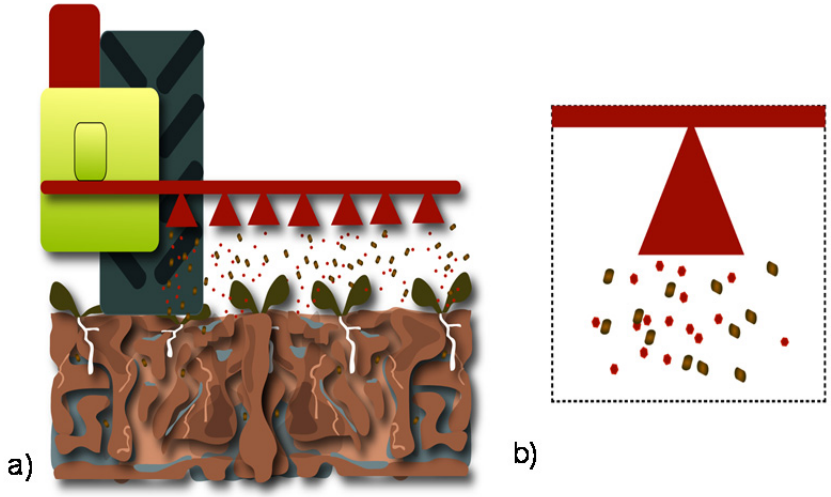

Figure 2. a) The bioprophylactic concept with simultaneous application of pesticide and pesticide degrader. b) A close-up showing pesticide (red) and pesticide degrader (green) (illustration by J. Forslund).

Enhanced pesticide degradation by inoculation with degrading microorganisms is not new, and has been extensively studied for bioremediation purposes (Megharaj *et al.*, 2011; Grundmann *et al.*, 2007; Schroll *et al.*, 2004; Singh *et al.*, 2004). However, there are some difficulties in remediation of aged contaminants that have been in contact with soil for a long time. Schroll *et al*. (2004) point out that diffusion of the contaminants to the applied microorganisms is required for enhanced degradation to take place. In addition, the microorganisms applied must survive and remain active for extended periods of time in the natural ecosystem (Schroll *et al.*, 2004). However, our proposed concept is based on prevention (*prophylaxis*) of pesticide contamination rather than remediation (**II**). Hence, potential difficulties of pesticide ageing are of no concern in our concept, since the bioavailability of non-aged pesticides immediately after application is at its maximum. In addition, simultaneous application of pesticide and degrading microorganisms minimises the diffusion paths needed for their contact and the microorganisms only need to be viable and active for the intended short period for complete degradation to occur. It is therefore not necessary or even desirable for the introduced microorganism to survive in the ecosystem after the intended task has been fulfilled.

Practical implementation of the concept requires that costs are kept low throughout the entire manufacturing process. The first step is cultivation of the

microorganism on an industrial scale, where the main objective is to maximise the productivity, *i.e.* to obtain the highest possible cell density in a given volume within a short time, and to minimise costs by using inexpensive substrates. Under ideal conditions, our concept requires approximately 10^{14} viable cells ha^{-1} to reduce a field dose of 1 kg MCPA ha^{-1} within 24 h, or no more than about 10 L of fermentate containing 10^{13} cells L^{-1}. These figures show that it would be possible to obtain a product at a realistic price provided that the cells are active and efficient.

3.2 The model herbicide

3.2.1 Phenoxy acid herbicides

The phenoxyacetic acid herbicide MCPA (Figure 3a) was used as a model substance for developing the bioprophylactic concept. Phenoxyacetic acid herbicides, such as MCPA and 2,4-D, belong to a subgroup of the larger group of phenoxy acid herbicides. The structures of some compounds in this group are presented in Figure 3a-f. The phenoxy acids are weak organic acids with high water solubility and low sorption to soil particles (Fredslund *et al.*, 2008; Hiller *et al.*, 2008; Sørensen *et al.*, 2006; Jensen *et al.*, 2004; Haberhauer *et al.*, 2002), properties that make these compounds very mobile in soil and thus susceptible to transport and leaching from soil to surface waters and groundwater. Since the start of the national environmental monitoring programme for pesticides in Sweden, MCPA and other phenoxy acids have been among the most frequently found herbicides, although generally at low concentrations (Nanos *et al.*; Adielsson, 2009; Törnquist *et al.*, 2007; Kreuger, 1998).

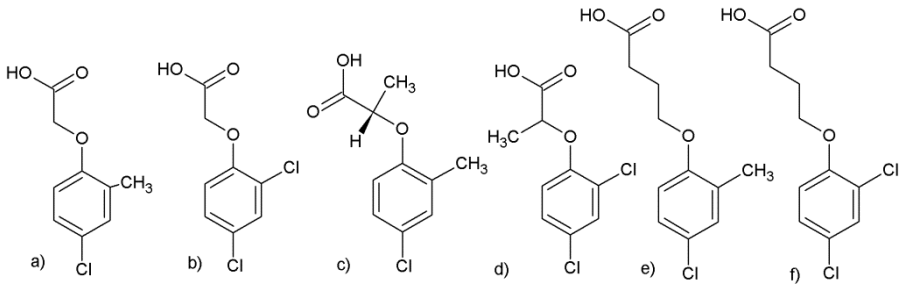

Figure 3. Structural formula of phenoxy acid herbicides with different carboxylic acids in their side chains. a) 4-chloro-2-methylphenoxyacetic acid (MCPA), b) 2,4-dichlorophenoxyacetic acid (2,4-D) c) (*R*)4-chloro-2-methylphenoxyproponic acid ((*R*)MCPP), d) 2,4-dichlorophenoxypropionic acid (2,4-DP), e) 4-chloro-2-methylphenoxybutaonic acid MCPB f) 2, 4-dichlorophenoxybutaonic acid (2,4-DB).

Phenoxy acids are systemic herbicides used for post-emergence control of annual and perennial broadleaved weeds in agriculture, as well as in non-agricultural applications. They are designed to mimic the function of the plant growth hormone auxin and stimulate the plant to overgrow until its death (Stenersen, 2004). These herbicides are absorbed through the leaves and are commonly used for the control of weeds in cereal crops, grass swards and lawns, individually or in combination with other active ingredients. In Sweden, pesticides with MCPA as the active ingredient, alone or in combination with others, are registered under the trade names Hormotex 750, MCPA 750 (formerly BASF MCPA 750) NUFARM MCPA 750, Ariane S and Stroller-Plus Trippel (KEMI, 2013b). In this thesis work, the formulation MCPA 750 (BASF MCPA 750) was used (**I**, **II** and field studies).

3.2.2 Microbial degradation of phenoxy acid herbicides

Microbial degradation is reported to be often the most important process for removal of phenoxy acid herbicides from the environment (Alexander, 1999). They are often metabolically degraded (**I**; Kilpi, 1980) and degraders can therefore be isolated through enrichment (**II**). The phenoxy acids 2,4-D and MCPA are rapidly degraded in agricultural topsoils (Sørensen *et al.*, 2006; Smejkal *et al.*, 2001c; Tett *et al.*, 1997; Hoffmann *et al.*, 1996), with the half-life of MCPA ranging from 1.5-16 days under aerobic conditions, while degradation rates in subsoils are lower (Sørensen *et al.*, 2006). In the present study, the half-life of MCPA in the fine soil of the railway embankment was 44.5 (±7.1) days and was reduced to 13.7 (±11.3) days if the soil was previously treated with MCPA (**I**).

The metabolic pathways of phenoxy acid degradation have been extensively studied using pure bacterial cultures. The first step is the cleavage of the ether linkage to produce the corresponding phenol (Fulthorpe *et al.*, 1995) initiated by oxygenases and encoded by *cadAB* or *tfdA*-like genes (Itoh *et al*., 2004, 2002; Kitagawa *et al*., 2002). The remaining steps in the degradation pathway are encoded by the *tfdB-F* genes (Don *et al.*, 1985). Figure 4 shows the degradation pathway of MCPA performed by the most extensively studied phenoxy acid degrader *Cupriavidus necator* (formerly *Alcaligenes eutrophus* JMP134) (Don *et al*., 1985; Pieper et al., 1988; Ledger et al., 2006). *C. necator* harbours the transmissible plasmid pJP4, which contains all the genes necessary for enzymatic degradation of phenoxyacetic acids and related compounds (Don & Pemberton, 1985).

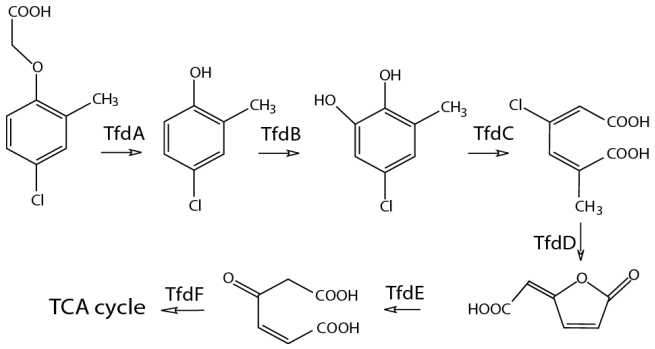

Figure 4. Degradation pathway of MCPA in *C. necator* JMP134 encoded by the *tfd* genes (modified from Perez-Pantoja *et al*., 2008).

Numerous strains capable of degrading phenoxy acids have been isolated from various environments (Smejkal *et al*., 2001c; Itoh *et al.,* 2000; Vallaeys *et al*., 1998; Kamagata *et al*., 1997; Tonso *et al*., 1995; Don *et al*., 1985). These bacteria are categorised into three groups based on their evolutionary position (Itoh et al., 2004, 2002; McGowan *et al*., 1998). Group I consists of β- and γ-subdivisions of proteobacteria that harbour *tfdA*-like genes (*i.e*. *tfdA or tfdA*α). This group can be further divided into three classes (classes I to III) based on their *tfdA* gene sequences, with *C. necator* belonging to class I (McGowan *et al.*, 1998). The second group includes the cluster of α-proteobacteria belonging to the *Bradyrhizobium* (or related organisms), while group III consists of α-proteobacteria closely related to *Sphingomonas* organisms (Zaprasis *et al.*, 2010; Kamagata *et al.*, 1997; Fulthorpe *et al.*, 1995). Group II and III organisms harbour both *tfdA*-like and *cadAB* genes (Zaprasis *et al.*, 2010; Itoh *et al.*, 2004, 2002; Kitagawa *et al.*, 2002).

3.3 The model microorganism

3.3.1 Enrichment and isolation of MCPA-degrading bacteria

In this thesis, phenoxy acid-degrading microorganisms were isolated by enrichment on MCPA, using soil from a former herbicide production plant at Teckomatorp, Sweden (**II**). The history of Teckomatorp needs be mentioned in this context, as it was the site of Sweden's first major environmental scandal. During the 1970s, the chemical company BT Kemi dumped hundreds of barrels containing tonnes of soil contaminated with several different pesticides including phenoxy acids, with devastating consequences for the environment and for people living in the area. The event itself and the debate, investigations

and legal processes that followed resulted in a redefinition of what was regarded as criminal concerning environmental protection in Sweden. It ultimately led to the inclusion of a paragraph on environmental crime in the Swedish penal code in 1981 (BRÅ, 2013). Since that scandal was first uncovered, a number of efforts have been made to clean up the site, but new unexpected findings of contaminated soil and inadequate methods used have resulted in the area still being under remediation more than 35 years later.

From this soil, which is highly contaminated with phenoxy acids, a MCPA-degrading consortium was enriched (**II**). A number of strains were isolated from the mixed culture by sub-culturing on agar plates containing MCPA to obtain single colonies. None of the isolated strains was capable of degrading MCPA in pure culture. However, MCPA degradation took place in different combinations of the isolates. Subsequently, it was found that only one strain, designated T51, had the ability to degrade MCPA in pure culture if grown on a complex medium or on a minimal salt (MS) medium complemented with casamino acids (CA). This strain was therefore selected for subsequent studies.

3.3.2 Identification of strain T51

Strain T51 was first deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ) (accession number DSM-No.18140) for a patent procedure and was determined to be most probably a new species within the genera *Sphingomonas*. This is also the classification used in Paper **II** and in the work by Wessman *et al.* (2011). Later it was found that this suggestion was based on a very short 16S sequence. It was also found that strain T51 did not reduce nitrate to nitrite, a characteristic feature of all members of *Sphingomonas* (Takeuchi *et al.*, 2001). After a second sequencing, using a major part of the 16S rRNA genes (1337 bp), strain T51 was identified to belong to the genera *Sphingobium* (**III**). The closest sequence match to the type strains using the analysis tool Seq Match at Ribosomal Database Project (RDP) was *Sphingobium xenophagum* BN6^T (Pal *et al.*, 2006). The sequences of strain T51 and *S. xenophagum* BN6^T (GenBank accession number NR_026304) were aligned using ClustalW (Larkin *et al.*, 2007), and showed 99.0% identity. Hence, strain T51 was re-identified as a *Sphingobium* sp. and was submitted to National Center for Biotechnology Information (NCBI) with the accession number KC136353. A phylogenetic tree was constructed using 16S rRNA gene sequences of strain T51 and the 20 most closely related type strains found in RDP. Sequences were aligned to the SILVA database (http://www.arb-silva.de/) using the SINA Webaligner (Pruesse *et al.*, 2012) and the phylogenetic tree was constructed based on the maximum-likelihood

algorithms PHYML (Guindon & Gascuel, 2003) in the sequence analysis software Geneious 6.1.2, Biomatters, New Zealand (http://www.geneious.com/). It was found that strain T51 most closely clustered with *Sphingobium xhenophaga* BN6^T (Figure 5). However, the tree was characterised by limited robustness, with only a few nodes supported by bootstrap values (based on 500 replications) >50%. This indicates that the relative position of each strain can vary within the cluster if the tree is rebuilt.

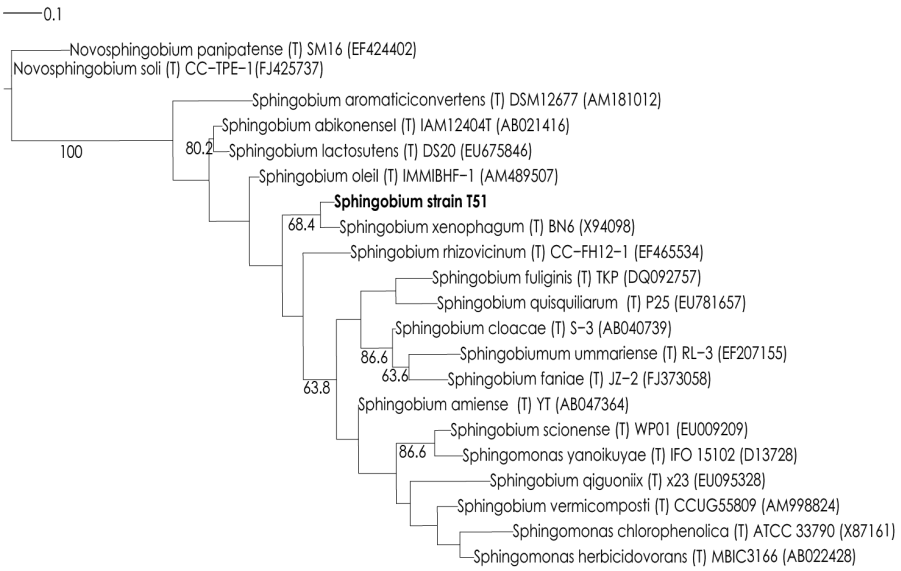

Figure 5. Phylogenetic tree based on 16S rRNA sequences from strain T51 and its closest relatives (type strains) using the maximum-likelihood algorithm PHYML. Bootstrap values (%) based on 500 replicates are shown for branches with >50% bootstrap support. Scale bars indicate 0.1 nucleotide substitutions per site.

3.3.3 Sphingomonads

The genus *Sphingomonas* was separated from *Pseudomonas* by Yabuuchi *et al*. (1990) and further classified by Takeuch *et al*. (2001) into four genera: *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*. However, this division was later rejected by Yabuuchi *et al.* (2002). The position of the genera *Sphingobium*, *Novosphingobium* and *Sphingopyxis* is currently under debate and most *Sphingomonas* are not yet classified accordingly. To avoid taxonomical confusions of the genus, *Sphingomonas* is defined *sensu latu* (including *Sphingobium*, *Novosphingobium*, *Sphingomonas* and *Sphingopyxis*) in this thesis and the term sphingomonads is used to cover all genera belonging to this group. However, the more specific genus names are used when a

classification is available. Sphingomonads are strictly aerobic, chemoheterotrophic, Gram-negative, rod-shaped and usually yellow-pigmented bacteria. The genus includes species that were originally described as members of the genera *Pseudomonas*, *Flavobacterium* and *Arthrobacter* (Yabuuchi *et al.*, 1990). Phylogenetically, the genus belongs to the α-4 subgroup of the proteobacteria and clusters together with *Blastosomas*, *Rhizomonas*, *Erythobacter*, *Erythromicrobiuim*, *Erythromonas*, *Porphyrobacter*, *Sandaracinobacter* and *Zymomonas* (Takeuchi *et al.*, 2001).

Sphingomonads degrade a range of organic compounds and are widely distributed in nature, where they are most frequently found in soils and aquatic environments. Among the compounds that can be degraded by various sphingomonads are phenolic compounds (Corvini *et al.*, 2006; Ohta *et al.*, 2004; Fujii *et al.*, 2001; Arfmann *et al.*, 1997; Rutgers *et al.*, 1997), aromatic hydrocarbons (Sipila *et al.*, 2010; Shi & Bending, 2007; Cunliffe *et al.*, 2006; Uyttebroek *et al.*, 2006; Baraniecki *et al.*, 2002; Shi *et al.*, 2001), naphthalene sulphonates (Stolz *et al.*, 2000), phenyl ureas (Sørensen *et al.*, 2001) and phenoxy acids (Muller *et al.*, 2004; Park & Ka, 2003; Smejkal *et al.*, 2001a; Horvath *et al.*, 1990; Kilpi, 1980). Ka *et al.* (1994) found that sphingomonads are the dominant phenoxy acid degraders in certain soils. Sphingomonads contain glycosphingolipids in their outer cell membranes instead of lipopolysaccharides, which are usually the major components of the membranes of Gram-negative bacteria. Glycosphingolipids are also ubiquitous in eukaryotic cell membranes. The possession of glycosphingolipids makes hydrophobic compounds more accessible to cells, which is one of the reasons why sphingomonads are potentially useful for bioremediation purposes (Kawahara *et al.*, 2000).

3.3.4 *Sphingobium* sp. T51

As mentioned previously, *Sphingobium sp*. T51 can metabolise MCPA in MS medium in the absence of other carbon sources, if supplemented with CA. Addition of various mixtures of amino acids to the MS medium was tested to replace the CA (Table 2). MCPA degradation was only found using mixture 3. None of the individual amino acids in mixture 3 supported growth of strain T51 on MCPA, but a combination of L-arginine and L-methionine was sufficient. These findings are similar to results presented by Sørensen *et al*. (2002), who describe an auxotrophic isoproturon-degrading *Sphingomonas* sp. that required methionine or components supplied by association with other soil bacteria. Neither L-arginine nor L-methionine could be used as carbon source for growth of strain T51.

Table 2. *Amino acid mixtures tested for growth of Sphingobium sp. T51 on MCPA*

Mixture 1	***Mixture 2***	***Mixture 3***	***Mixture 4***
L-glutamic acid	L-phenyalanine	L-arginine	L-cysteine
L-proline	L-isoleucine	L-serine	L-tryptophan
L-lysine	L-aspartic acid	L-histidine	L-tyrosine
L-valine	L-alanine	Glycine	L-glutamine
L-leucine	L-theonine	L-methionine	L-aspergine

The majority of the strains capable of degrading phenoxy acids have been isolated using 2,4-D as substrate. Therefore most of the research related to catabolic genes and classification of degrader strains has been done based on these strains. MCPA has been less studied and few bacterial degraders have been isolated based on this compound (**II**; Gozdereliler *et al.*, 2013). 2,4-D and MCPA are structurally very similar and 2,4-D degraders commonly also degrade MCPA (Ka *et al.*, 1994), but with considerably slower degradation rates (Bælum *et al.*, 2010). In addition, MCPA has been shown to be more difficult to degrade than 2,4-D in the soil environment (Bælum *et al.*, 2008). *Sphingobium* sp. T51 is able to degrade 2,4-D, alone or in combination with MCPA (Figure 6). In contrast to what was found by Bælum *et al*. (2008), the two compounds were degraded simultaneously at identical rates, indicating use of the same degradation pathway and that T51 cells do not differentiate between MCPA and 2,4-D. In the study by Bælum *et al.* (2010), 2,4-D degraders were favoured by the use of 2,4-D as substrate during the isolation process, which possibly explains the difference in degradation rates. *Sphingobium sp.* T51 was also able to use the (*R*)-enantiomer of MCPP as a sole carbon source, while the (*S*)-enantiomer, MCPB and 2,4-DB were not degraded (structures are shown in Figure 3). In general, most isolated phenoxy acid degraders are restricted in their substrate utilisation abilities of related compounds (Ka *et al.*, 1994), but substrate specificity is not related to type of degradation genes (*i.e*. *tfdA* or *tfdA*-like genes) (Smejkal *et al.*, 2001b).

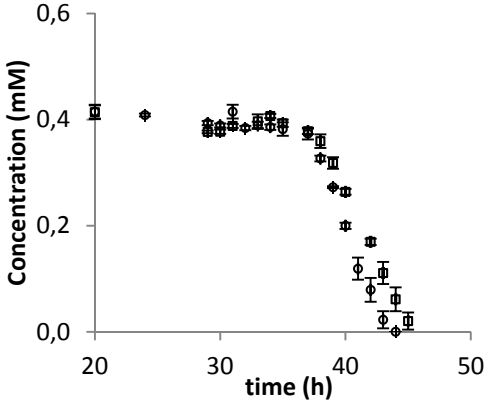

Figure 6. Simultaneous degradation of MCPA (○) and 2,4-D (□) in mixture by strain *Sphingobium* sp. T51.

MCPA at 50 mg L^{-1} was reduced by strain T51 to a level undetectable by HPLC (<0.05 mg L^{-1}) within 48 h in MS medium initially containing 0.15 mg CA L^{-1}. Provision of an additional carbon source has been reported either to enhance the biodegradation rate of pollutants by providing carbon and energy for supplementary growth of the degrading bacteria (Cheyns *et al.*, 2012; Hess *et al.*, 1990), or to repress the degradation through competitive inhibition (Lovanh *et al.*, 2002; Muller *et al.*, 1996). For strain T51, the addition of an extra carbon source such as glucose or acetate or a complex medium such as tryptic soy broth (TSB) improved cell growth and shortened the lag phase and the MCPA degradation time.

The temperature range for growth of *Sphingobium* sp. T51 on glucose (0.5 g L^{-1}) or sodium acetate (0.15 g L^{-1}) was 2-30 °C, with an optimum at 20-25 °C and complete growth inhibition at 35 °C. The same result was observed with rich medium (TSB 1/3 strength). MCPA degradation occurs at 2 °C, but is low, even in the presence of an additional carbon source. At 35 °C, degradation of MCPA began but stopped before less than 50% of the initially applied MCPA had been degraded. The ability of T51 cells to degrade MCPA at low temperatures is of interest, considering the frequently low soil temperatures in Sweden.

3.4 Proof of principle

In order to mimic pesticide use on non-agricultural soils poor in microbial activity, degradation of MCPA in sand was studied (**II**). Soil inoculation with *Sphingobium sp.* T51 enhanced the degradation so that MCPA residues were

below the limit of detection in 1-3 days, while very low degradation rates were found in the uninoculated sand (**II**). No accumulation of the metabolite 4-chloro-2-methyl phenol (MCP) was detected.

For metabolically degraded compounds such as MCPA, inoculum size and the specific degradation rate influence how fast degradation occurs. Arshad *et al.* (2008) found that a minimum cell density is often required to achieve enhanced degradation and that the degradation rate often increases with inoculum size up to a certain concentration, after which further increases have no or even a negative effect. With the experimental set-up in Paper **II,** an inoculum size of 10^5 CFU of *Sphingobium* sp. T51 per g dw of soil was sufficient to obtain significantly enhanced degradation, with 83% of the initially applied MCPA degraded after 1 day. As degradation time is dependent on the initial MCPA concentration, the inoculum size was increased in **IV**.

However, if the degradation is too fast, the intended herbicidal effect could be reduced or even eliminated (Robertson & Alexander, 1994). The concept was therefore tested with plants present, where white mustard (*Sinapis alba*) represented a weed sensitive to MCPA and winter wheat the non-sensitive crop (**II**). MCPA degraders were applied immediately after the herbicide and the results showed that the bioprophylactic concept enhanced MCPA degradation without reducing the herbicidal effect. However, it should be noted that the MCPA degraders were applied in a water solution which was perhaps deposited mainly on the soil and not on plant surfaces, where the herbicide has its mode of action. The topic of delayed degradation in this context is discussed in section 7.2.2.

3.5 Field studies

3.5.1 Risk assessment

Before considering up-scaling of the concept from laboratory experiments to field trials, the safety aspects have to be considered. For applications where microorganisms are released in the environment, safety assessments and consequence analyses need to be done to ensure that the organism is harmless both for people handling it and for the environment where it is released. Ideally, the microorganism should be evaluated shortly after isolation and identification to test that it fulfils basic safety requirements. The first concern is whether the microorganism is pathogenic or infectious to humans and animals and such an initial safety assessment often comprises a literature search on the genus in general, to avoid the use of species from genera with known hazardous strains, as well as tests for antibiotic resistance pattern and

determination of temperature range for growth. Initial safety tests should also include *in vitro* tests with biosensor organisms to obtain information about toxicity and pathogenicity (Sundh *et al.*, 2011). At present, there is no universal tool to address the safety issues and the actual requirements of EU legislation are not adapted to the evaluation of microorganisms (Sundh *et al.*, 2012). In the case of *Sphingobium* sp. T51, initial tests showed that it does not grow at 37 ºC and a literature search together with tests on antibiotic resistance gave no cause for concern. Further safety assessment of an organism can include its persistence in the new environment (*e.g*. soil), dispersal, the effect on non-target organisms, disruption of essential ecosystem functions, competition with non-target organisms, the effect on the natural diversity of microorganisms and the risk to human health during production, storage and application (Sundh *et al.*, 2012; Johansen *et al.*, 2005; Winding *et al.*, 2004; Kluepfel, 1993). There are several techniques available to follow the fate of an introduced bacterial species, including real-time PCR (Holmberg *et al.*, 2009; Kim & Knudsen, 2008; Savazzini *et al.*, 2008) and different DNA fingerprinting methods, such as denaturing gradient gel electrophoresis (DDGE) (Niu *et al.*, 2009), and fluorescent *in situ* hybridisation (FISH) (Kleikemper *et al.*, 2002).

3.5.2 Field study on railway embankment

The first field experiment in which we tested the bioprophylactic concept was conducted on a railway embankment at Viskadalsbanan just north of Varberg (57°82'N, 12°16'E) during summer 2007. The embankment mainly consists of coarse sand, with stones in various sizes in the surface layer. Larger stones were removed from the surface before eight 1.5 x 1.5 m plots (4 controls and 4 inoculated) were established from the middle of the track outwards. Experimental plot vegetation was removed to allow uniform application. The herbicide was applied using a special train for spraying railway tracks, at a dose corresponding to 1.5 kg MCPA 750 ha^{-1}. The suspension of *Sphingobium* sp. T51 was applied using a hand-held pressurised sprayer to give 10^6 cells per cm^2 in the upper cm of soil. The embankment soil was sampled one and five days after pesticide application. The uppermost layer (0-30 cm) was sampled by cutting a sample with dimensions 5 cm × 5 cm and 5 cm depth with a tube-type soil sampler. The procedure was repeated for each of the layers to be sampled, with four replicates (chosen randomly) per plot at each depth. The replicates were pooled and all soil samples were frozen within 1 h of collection. They were later analysed for MCPA content using high-

performance liquid chromatography (HPLC) (for a description of the analytical method, see the Materials and Methods section in Papers **I** and **II**).

The enhanced MCPA degradation after inoculation with *Sphingobium* sp. T51 obtained in microcosm studies (**II**, **IV**) could not be repeated under field conditions (Figure 7). High variability in MCPA concentrations made the results difficult to interpret. First of all, and perhaps the most important finding, was the overall low concentration of MCPA in the control soil (Figure 7). Considering the half-life values found in ballast soil in Paper **I** and the fact that this track had previously not been exposed to MCPA, it is very unlikely that 68-96% of the applied MCPA would have been degraded within one day of application. Thus the reduction observed was probably not a result of degradation, but rather indicates further transport of the herbicide. Relatively heavy rain shortly after application most probably had a large influence on the fate of the herbicide. Although the highest concentrations of MCPA were mainly found in the upper 15 cm one day after application, the herbicide was detected in all samples taken at the lowest depth (25-30 cm) in concentrations ranging from 0.06-0.4 µg MCPA g^{-1}.

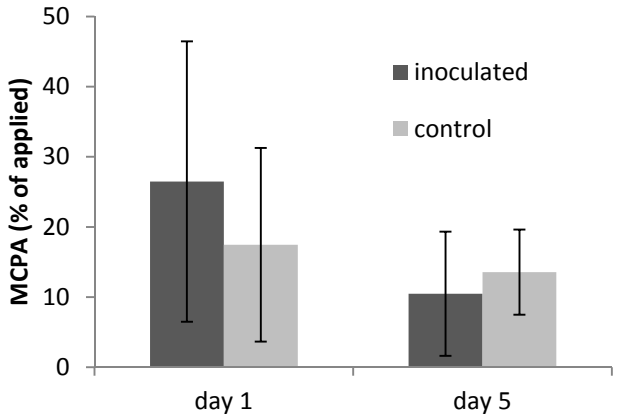

Figure 7. MCPA concentrations in ballast soil after herbicide application. Comparison between bioaugmentation with *Sphingobium* sp. T51 and non-inoculated controls.

The fate of *Sphingobium* sp. T51 after application was not investigated and its role in MCPA degradation in the ballast soil is not clear. MCPA degradation by strain T51 would be expected to occur primarily in the surface soil, but no significant differences in MCPA concentration were found between inoculated

and uninoculated control plots in the upper 5 cm samples (Figure 8), indicating negligible degradation activity of T51 cells. There was a temperature shift at the time of application, which possibly affected the degradation efficiency of the degraders. *Sphingobium* sp. T51 was cultivated and thereafter stored in phosphate buffer for two days before application at a temperature of 20-25 °C. However, the field temperature was 10 °C lower at the time of application. In further laboratory studies, it was found that the lag phase before MCPA degradation was longer if strain T51 was pre-cultured at a higher temperature (*i*.e. 20 °C) compared with pre-cultivation at the same low temperature as in the degradation experiments (*i.e*. 10 °C).

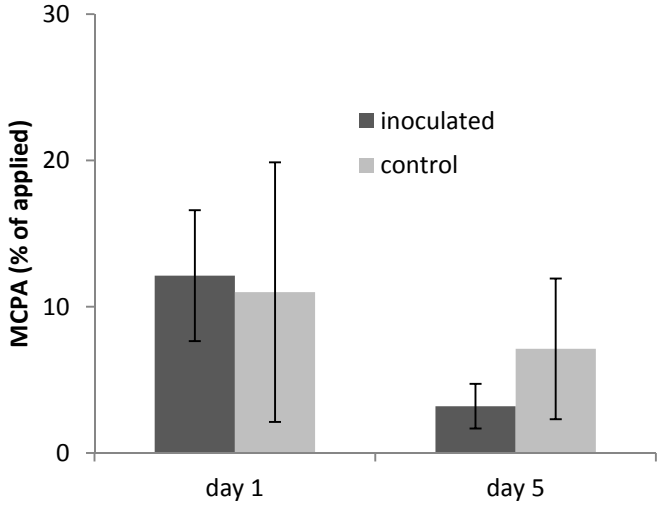

Figure 8. Remaining MCPA in the upper 5 cm of the ballast soil in the field study on the railway embankment after bioaugmentation with *Sphingobium* sp. T51 and in the non-inoculated control.

3.5.3 Field study on arable land

A second field study was conducted on an agricultural soil with no history of MCPA use at an experimental farm at Fransåker near Knivsta (59°39'N, 17°50'E). The soil in the study field is a sandy clay loam soil. An area of 25 m x 100 m was selected and divided into five plots. The field was tilled and harrowed before white mustard was sown on three plots. MCPA (MCPA 750, 2.5 kg a.i. ha^{-1}) was sprayed three weeks after sowing, leaving two of the plots with white mustard as controls without pesticide application (of which one was also a control without bacteria inoculation). A suspension of *Sphingobium* sp. T51 was applied using a sprayer (3-m boom with six nozzles) on three plots

(Figure 9), one with bare soil (for soil sampling and MCPA analysis), one with plants and MCPA (for weed control) and one with plants as a control without MCPA. The evenness of distribution of T51 cells was determined by serial dilutions and spread counts from petri dishes placed randomly in the field at the time of bacteria application. The two plots without plants were divided into 10 blocks and soil samples were taken according to a randomised scheme in the 0-5 cm and 5-10 cm layers using a tube-type soil sampler at start and at days 1 and 3. All soil samples were frozen within 1 h after collection and were analysed for MCPA content using HPLC (**I** and **II**).

Figure 9. Field trial on arable land. Upper left: Spraying of *Sphingobium* sp. T51. Upper right: Preparation of inoculum prior to application. Lower left: White mustard after application of MCPA and *Sphingobium* sp. T51. Lower right: Control with white mustard without herbicide application.

The results from the field study on arable soil were similar to those of the first field study (Figure 10), but the weather conditions also included drought and high temperatures that were detrimental for the efficacy of the system. The direct exposure to UV-radiation of the unformulated organism probably also contributed to the lack of enhanced MCPA degradation (Cohen & Joseph, 2009; Ragaei, 1999). From visual observations, the herbicide effect was determined as good even where the degrader was applied (Figure 9).

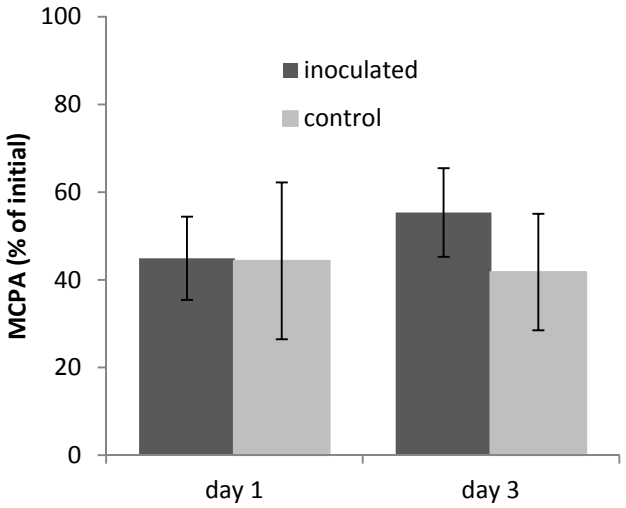

Figure 10. Remaining MCPA in arable soil after bioaugmentation with *Sphingobium* sp. T51 and in the non-inoculated control.

3.5.4 Conclusions from field studies

Unpredictable weather conditions together with non-optimised bacterial inocula did not allow proof of the bioprophylactic concept to be obtained in the field. Clearly, the presence of *Sphingobium* sp. T51 had no positive effect on MCPA degradation and questions on the robustness of the concept were inevitably raised. However, the lack of enhanced degradation in the field is not surprising. In fact, the number of reports on successful field trials is small, despite numerous experiments using potentially effective microorganisms for bioremediation purposes or as biocontrol agents (Santoyo *et al.*, 2012; Tyagi *et al.*, 2011). Under laboratory conditions microorganisms can function optimally, but it is difficult to obtain equivalent results in field applications. One of the greatest barriers to successful use of bioaugmentation in the field is therefore a lack of reliable methods and procedures that consistently give the desired activity and efficiency (Tyagi *et al.*, 2011). This is also one of the major obstacles to the commercialisation of microbe-based products. One step towards a solution to these problems could be formulation of the microorganisms, both to achieve long-term stability during storage after cultivation and functionality in the field.

4 Formulation of *Sphingobium* sp. T51

In formulation of microorganisms, several factors must be considered at all stages from production of the microorganism of interest to its application. General requirements on microbial formulations are that they must be stable during storage, maintain the biological activity, be cost-effective and give a product that is easy to handle and use for both producers and end-users (Melin *et al.*, 2007). In order to formulate pesticide degraders for the bioprophylactic concept, it is essential to find a formulation that, in addition to these general requirements, also protects the microorganism from harmful environmental factors during and after application in the field and provides optimal conditions for pesticide degradation.

There are many different types of formulations: dry products such as wettable powders, dusts and granules, and liquid products such as cell suspensions in water and oil emulsions. Dry formulations are often used to enhance the storage stability and to give a product that is easy to handle (Santivarangkna *et al.*, 2007) and such formulations were the main focus in our efforts to formulate *Sphingobium* sp. T51 (**III**, **IV**). In Paper **III** we investigated the role of different formulation excipients, with the aim of obtaining some more general information on different processes taking place in the bacterial cell during dehydration and subsequent storage. Paper **IV** dealt with more strain-specific aspects concerning the formulation of *Sphingobium* sp. T51 for efficient use in the bioprophylactic concept.

4.1 Dry formulations

Preservation of microorganisms by desiccation has been used for decades for preserving culture collections and within the food and pharmaceutical industries. In spite of the widespread need for drying technologies and the

many different methods used, there is surprisingly little generic open knowledge within the area (Fu & Chen, 2011; Santivarangkna *et al.*, 2007; Morgan *et al.*, 2006). Therefore, the best formulation for a particular microorganism is still mainly determined empirically by trial-and-error. In addition, formulation of beneficial bacterial strains is often associated with product development on an industrial scale and therefore also with patent issues, and consequently formulation compositions and process parameters are often proprietary information.

The preservation of bacterial cells through drying is based on a reduction in the cell metabolic rate by removing or decreasing the amount of available water (Potts, 2001). Drying is considered to be extremely stressful to the bacterial cell, primarily due to changes in membrane lipids and protein structure, which can lead to severe loss of cell viability (Potts, 2001). The microorganisms can respond to the stress either by attempts to resist it or by adaptation (Franks *et al.*, 1990). The adaptive response to desiccation includes shifts in membrane lipid composition (Schoug *et al.*, 2008), changes in the synthesis of stress proteins (Baati *et al.*, 2000) and uptake of compatible solutes (Bergenholtz *et al.*, 2012; Kempf & Bremer, 1998).

Although there are several different techniques for drying microorganisms, of which freeze drying, spray drying and fluid bed drying are among the most commonly used (Santivarangkna *et al.*, 2007), the process comprises certain general steps as shown in Figure 11.

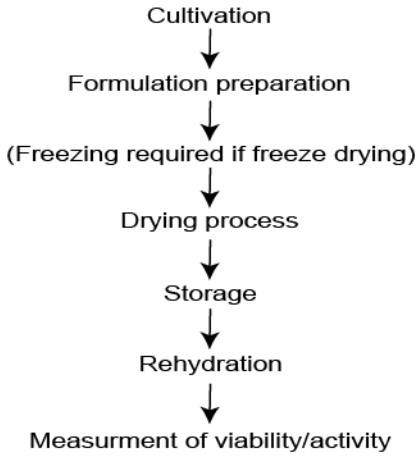

Figure 11. General drying process for microorganisms (modified from Morgan *et al.*, 2006).

4.1.1 Freeze drying

Freeze drying is a well-studied method used for food, pharmaceutical and biotechnological applications. The freeze drying process can be divided into three steps: an initial freezing, followed by primary and secondary dryings. The amount of available liquid water is reduced during freezing by the formation of ice crystals. The rate of freezing controls the concentrations of solutes around the cell and influences cell survival (Schoug *et al.*, 2006). During the primary drying, ice crystals are removed by sublimation due to the low pressure used. The secondary drying aims to remove any unfrozen water not removed in the primary drying and is needed for adequate storage stability (Morgan *et al.*, 2006). It is debated whether over-drying, *i.e*. removal of too much water, affects cell survival rates (Passot *et al.*, 2012; Zayed & Roos, 2004), but over-drying was at least not found to be crucial for the initial survival rates of *Sphingobium* sp. T51 (**III**).

The aim of this part of the thesis work was not to optimise the freeze drying process for *Sphingobium* sp. T51, so it was freeze-dried in a pilot plant freeze dryer (Lyostar II, FTS kinetics, USA) using a conventional method previously developed by our group for freeze drying of lactic acid bacteria (Schoug *et al.*, 2010).

4.1.2 Fluidised bed drying

In fluidised bed drying, a jet of usually heated air or gas blows through a bed of solid particles at a rate high enough to set them in motion. The system has a high efficiency due to the close contact between the gas and the wet particles (Mille *et al.*, 2004) and is used mainly in the food processing, pharmaceuticals and chemical industries (Horaczek & Viernstein, 2004). Depending on the temperature, which can be varied widely, fluidised bed drying is a rapid process (from 1 min to 2 h; Santivarangkna *et al.*, 2007) compared with freeze drying (36 h; **III)**. Although fluidised bed drying of microorganisms has been implemented with high survival rates and sufficient storage stability (Stummer *et al.*, 2012; Fu & Chen, 2011; Selmer-Olsen *et al.*, 1999; Bayrock & Ingledew, 1997), and is used for large-scale production of baker's yeast (*Saccharomyces cerevisiae*) (Bayrock & Ingledew, 1997), it has not yet received widespread application (Santivarangkna *et al.*, 2007; Morgan *et al.*, 2006). Only materials that can be granulated are dryable and microorganisms must therefore be mixed with a carrier material prior to fluidised bed drying. This can result in agglomeration of particles of varying sizes during drying, sometimes with a sticky nature, which decreases the drying rate and negatively affects storage stability (Santivarangkna *et al.*, 2007). This effect was

occasionally observed during the fluidised bed drying of strain T51 and was found to be dependent on the carrier material used.

4.2 Formulation excipients and their protective effects

The initial survival rate of *Sphingobium* sp. T51 using either freeze drying or fluidised bed drying was high (60-85%) (**III**, **IV**), indicating that the strain has an inherent good tolerance to drying. However, freeze drying of cell pellets of T51 without a protective agent resulted in low cell survival rates. Sufficient cell viability is dependent on the addition of protective agents or carrier materials prior to the drying process to reduce some undesirable effects of dehydration (Morgan *et al.*, 2006). Many different formulation excipients have been used as protective agents. Addition of *e.g*. monosaccharides, disaccharides, sugar alcohols, skimmed milk, peptones and amino acids has been found to be effective for stabilising cell membrane structure and proteins during both dehydration and storage (Schoug *et al.*, 2010; Morgan *et al.*, 2006; Carvalho *et al.*, 2002; Franks *et al.*, 1990). However, the selection of an optimal formulation excipient is not an easy task, as the effect of different protective agents and carriers during and after drying appears to be strain-specific and dependent on the dehydration technique used. Consequently, there are still many unanswered questions as regards the mechanisms behind the protective and stabilising effects.

4.2.1 Protective excipients

It is well known that desiccation-tolerant microorganisms accumulate disaccharides intracellularly, primarily sucrose and trehalose, in order to protect subcellular structures vital for cell survival (Crowe *et al.*, 1998; Crowe *et al.*, 1994). Consequently, these sugars are extensively used as protectants to increase cell survival following dehydration. Disaccharides are believed to have stabilising effects on lipid membranes and proteins by replacing hydrogen bonds otherwise lost upon drying (*i.e*. by substituting for water molecules) (Leslie *et al.*, 1995). In addition, they readily form an amorphous structure through a process called vitrification. The amorphous structure is considered to be a prerequisite for cell survival following freeze drying, although it is not necessarily sufficient *per se* (Bergenholtz *et al.*, 2012; Santivarangkna *et al.*, 2011; Crowe *et al.*, 1998). The amorphous state is of high viscosity with low molecular motility that reduces chemical rearrangement of the material (Crowe *et al.*, 1998; Crowe *et al.*, 1988). Further, the amorphous structure is suggested

to be important in order to embed, shield and physically separate the cells (Santivarangkna *et al.*, 2011; Wessman *et al.*, 2011; Oliver *et al.*, 1998).

Other compounds, for instance organic polymers, also readily vitrify and become amorphous upon removal of water. Polymers are normally used as formulation additives to other freeze drying excipients (*e.g.* disaccharides) (Schoug *et al.*, 2010; Devaldez *et al.*, 1983) and their use as major formulation constituents for microbial preservation is uncommon (Wessman *et al.*, 2011). It has been suggested that disaccharides are needed both in the intracellular compartment and in the extracellular milieu for optimal stabilisation in the dry state (Billi & Potts, 2002; Leslie *et al.*, 1995). However, freeze drying survival rates of bacterial cells formulated with organic polymers have been found to be comparable to those achieved when using formulations based on non-reducing disaccharides (**III**; Wessman *et al.*, 2011). The polymers are large molecules and are not likely to be taken up by the bacterial cell, and would thus not have the capacity for water-replacing effects that non-reducing disaccharides are suggested to have (Crowe *et al.*, 1997; Crowe *et al.*, 1994). In Paper **III**, intracellular sugar analysis (performed by high resolution magic angle spinning nuclear magnetic resonance, HR-MAS-NMR) showed that neither of the disaccharides sucrose or trehalose could be detected in cells prior to formulation, but that trehalose was present in both the polymer and disaccharide samples after formulation and freeze drying. This indicates that the accumulated trehalose is formed after harvest but prior to freeze drying (*i.e.* during formulation), and was suggested to be sufficient for cell protection during the dehydration process in this case (**III**).

Protectants of a more complex nature can also be used as additives in the drying of microorganisms. For example, several studies have shown skimmed milk powder to be an effective protectant mainly for storage stability (Horaczek & Viernstein, 2004; Champagne *et al.*, 1996; Castro *et al.*, 1995). In the present study, skimmed milk powder had a slightly negative effect on the initial survival rates for strain T51 during fluidised bed drying, but increased storage survival under certain conditions (**IV**).

4.2.2 Carriers

The carrier needed to achieve a solid material during fluidised bed drying not only granulises cells, but also provides protection for them during drying and subsequent storage. Moreover, carrier-based formulations are commonly used for preparation of microbe-based products used for soil inoculation. The carrier enables easy handling of the product, but also acts as a protectant to enhance efficacy in the field. The choice of carrier largely depends on the

microorganism and the application, but a suitable carrier should be non-toxic to the organism, easy to process, inexpensive, have good pH buffering capacity, and be non-toxic to the environment of application (van Veen *et al.*, 1997). Various types of materials are used as carriers for soil inoculation, with peat being the most commonly used (Ben Rebah *et al.*, 2007; Walker *et al.*, 2004; Stephens & Rask, 2000). Peat was found to be ineffective in protection of *Sphingobium* sp. T51 during fluidised bed drying. This was first thought to be caused by the low pH of the peat. Increasing the pH increased cell survival rates, but not by enough to be acceptable (*i.e.* <0.1% of initial cell density). Alternative carriers for soil inoculations, of both inorganic and organic origin, have been investigated (Stephens & Rask, 2000). Inorganic clay carriers have been shown to improve cell survival rates of root nodule bacteria in the field (England *et al.*, 1993; Marshall, 1968). The clay particles act as a bulking agent and form a protective envelope around the cells, providing mainly physical protection (Cassidy *et al.*, 1996; Marshall, 1968). In addition, a formulation including the clay vermiculite has been successfully used for fluidised bed drying of a biocontrol strain (Moenne-Loccoz *et al.*, 1999).

In many field applications, the survival in soil of the inoculated bacteria has to be taken into account. Formulations containing nutrient amendments may be useful in enhancing survival and efficacy after soil inoculation of microorganisms (Schisler *et al.*, 2004; van Veen *et al.*, 1997). For the bioprophylactic concept, long-term survival in the field is not required or even desired. Nevertheless, it may be of interest to use a carrier material that can support initial growth, in order to achieve fast pesticide degradation (**IV**). Cottonseed flour, like peat, is an organic carrier and has been used for the biocontrol yeast *Pichia anomala* J121 with good survival rates and storage stability after fluidised bed drying (Melin *et al.*, 2007). Cottonseed flour is a complex medium that, in addition to its role as carrier material, may also offer essential substances or act as an additional growth substrate for the inoculated degrader in the field (**IV**). In addition, different plant by-products, composted plant materials, animal manure and charcoal have been used as carriers for soil inoculation (Stephens & Rask, 2000). However, their role as a bulk material for fluidised bed drying has not yet been investigated.

5 Storage stability

5.1 Cell viability

One of the main reasons for using a dry formulation is to enhance the shelf-life of a microbial product. The desired shelf-life of a microbial product is often one year, preferably at room temperature. Strain T51 survives both the freeze drying and fluidised bed drying process well (**III**, **IV**), but the ability of a formulation to support a high initial cell survival is no guarantee of good shelf-life. Oxygen, moisture, light, microbial contamination and high temperatures during storage are factors that can negatively affect formulation stability (Santivarangkna *et al.*, 2008; Morgan *et al.*, 2006; Carvalho *et al.*, 2004). In addition, storage stability is dependent on pre-formulation conditions (*e.g.* growth medium, time of harvest and cell density) and protective excipient/carrier used (Morgan *et al.*, 2006; Schoug *et al.*, 2006; Carvalho *et al.*, 2004). Storage stability differs between species and strains and, again, it is not easy to make generalisations. However, survival rates of Gram-negative bacteria during storage tend to be lower than those of Gram-positive bacteria and this has been suggested to be due to differences in cell envelope structure (Miyamoto-Shinohara *et al.*, 2008).

Cell survival of strain T51 after fluidised bed drying was inversely related to storage temperature, in accordance with other reports (Strasser *et al.*, 2009; Champagne *et al.*, 1996), but was also influenced by formulation composition (**IV**). However, a storage temperature of 25 °C was detrimental for fluidised bed-dried T51 cells, irrespective of the formulation used. In contrast, freeze-dried T51 cells had acceptable survival rates at 25 °C, at least if stored under partial vacuum conditions (**III**). T51 cells freeze-dried in sucrose or Ficoll resulted in equally high cell survival rates of approximately 50% during the first three months of storage, after which Ficoll continued to lose in cell

viability whereas the sucrose-based formulation had stabilised (**IV**). Paper **III** investigated the influence of different atmospheric conditions on the storage stability of strain T51. It found that although the polymers Ficoll and HEC were well suited to support cell survival during the freeze drying process and the Ficoll formulation performed well during storage in vacuum, these formulations were incapable of impeding detrimental effects of oxygen and moisture exposure during storage. This indicates that disaccharides have protective properties that are of importance during storage, which the polymers lack. The intracellular trehalose that was formed in T51 cells during the formulation procedure mentioned earlier, or other components, possibly served as a basic protective agent during freeze drying and subsequent storage under vacuum conditions. However, under non-vacuum conditions, with the additional external stressors oxygen and moisture, this protection was not sufficient (**III**).

It is also likely that the generally low survival rates during storage of the fluidised bed-dried material (**IV**) were due to the storage atmosphere (*i.e*. exposure to air) and could thus be improved by using more favourable storage conditions (*i.e*. nitrogen or vacuum atmosphere). Shifting the atmosphere from air to nitrogen during storage has been found to increase bacterial survival (Kurtmann *et al.*, 2009; Castro *et al.*, 1995) and storage under vacuum is reported to significantly enhance storage stability of several bacterial strains (Hernandez *et al.*, 2007; Castro *et al.*, 1995; Bozoglu *et al.*, 1987). In addition to storage atmosphere, increased cell survival rates have also been found after fluidised bed drying of strain *Lactobacillus bulgaricus* under a flow of nitrogen gas compared with drying under air flow (Mille *et al.*, 2004). However, this is not expected to influence the viability of *Sphingobium* sp. T51 considering the high initial cell survival rates already obtained (**IV**).

The amount of water remaining in the samples was on average 5-7% for the fluidised bed-dried formulations, compared with 1.5-2% for the freeze-dried formulations (**III, IV**). However, higher water content may not necessarily result in lower storage stability (Passot *et al.*, 2012; Zayed & Roos, 2004). Zayed & Roos (2004) found that water contents of 2.8-5.6% improved cell survival rates during storage of a freeze-dried *Lactobacillus* strain. We cannot draw any conclusions on the effect of water content on cell survival of *Sphingobium* sp. T51, since the dehydration techniques and storage conditions used are not comparable (*i.e*. different formulations, storage atmospheres and temperatures). However, it is possible that the overall poor storage stability of the freeze-dried HEC formulation was related to its very low water content

(0.3%) (**III**). Negative effects on bacterial viability have been observed at moisture contents close to zero (Passot *et al.*, 2012; Zayed & Roos, 2004).

The addition of skimmed milk powder had a negative effect on the initial survival rates when combined with cottonseed as carrier (**IV**), possibly due to the fact that this mixture resulted in a paste that did not pulverise easily during drying. However, skimmed milk powder gave some positive effects on survival rates during storage for the formulation with cottonseed flour, but the effect was only observed at 4 °C and only after four weeks of storage (**IV**). The reason for the stabilising effect is not fully understood, but it is suggested that skimmed milk proteins form a protective coating on the cell walls and thereby increase the survival rate during storage (Abadias *et al.*, 2001).

5.2 Subcellular damage

Microbial formulations are generally evaluated based on cell survival rates, or at most their efficacy (*e.g.* maintenance of biological activity) (see Chapter 6). Attempts have been made to investigate subcellular damage, in order to understand the mechanisms causing cell death during dehydration and subsequent storage, but present knowledge is based on relatively few studies (reviewed by Fu & Chen (2011) and Garcia (2011)). The impairment of viability during storage is suggested to mainly be related to oxidative stress and subsequent damage to the cell membranes by formation of reactive oxygen species (ROS), which in turn cause lipid peroxidation (Santivarangkna *et al.*, 2008; Linders *et al.*, 1997; Teixeira *et al.*, 1996), protein denaturation (Fredrickson *et al.*, 2008; Franca *et al.*, 2007; Potts, 1994) and DNA damage (Teixeira *et al.*, 1995b). In a metabolically active cell, these ROS are trapped by the antioxidant defence system, but during prolonged storage in the dry state, the cell membranes are more susceptible to ROS attack (Franca *et al.*, 2007). Antioxidants have been shown to reduce oxidation-related damage and to prolong storage stability (Kurtmann *et al.*, 2009; Teixeira *et al.*, 1995a) but in other studies, including this thesis, this effect could not be observed (**III**; Nag & Das, 2013). On the other hand, we were unable to detect lipid peroxidation, despite severe losses in storage stability in the presence of air, indicating that lipid peroxidation is not a controlling factor leading to cell death in this system (**III**).

We also measured the RNA integrity number (RIN) (Schroeder *et al.*, 2006) to analyse RNA quality, *i.e.* RNA degradation (**III**). RNA has not received much attention as a target for intracellular oxidation processes to date, but is suggested to be more susceptible to oxidative damage than DNA (Hofer *et al.*,

2005). Interestingly, the results indicate that RNA was unaffected by the freeze drying process in all formulations and no apparent connection between RNA quality and bacterial survival rates could be found during storage. However, the freeze-dried polymer formulations stored under humid conditions gave the lowest RIN values. It was suggested that exposure to moisture leads to increased cellular RNA degradation and that such an event contributes to controlling cell survival, but is not necessarily a decisive factor, and that disaccharides possibly have a protective effect on RNA integrity.

6 Degradation efficiency and leaching

6.1 Degradation efficiency in microcosms

An appropriate formulation should not only maintain high bacterial survival during drying and storage, but also retain the effectiveness of the microorganism, *i.e*. the biological activity (Nopcharoenkul *et al.*, 2011; Melin *et al.*, 2007). Selective loss of plasmid-encoded phenotypic expression has been shown during freeze drying (Lange & Weber, 1995). Moreover, viable cells may suffer sub-lethal injury after drying and rehydration, resulting in a need for extra time to repair the injury (*i.e*. prolonged lag phase) before starting to grow as normal cells (Teixeira *et al.*, 1995a). All T51 formulations maintained their degradation efficacy in soil microcosms and performed at least equally well as fresh T51 cells with regard to MCPA degradation when equal numbers of viable cells were used (**IV**). Inoculation with non-viable cells did not enhance MCPA degradation rates compared with uninoculated controls.

However, one observation made during viability measurements (plate counts) of fluidised bed-dried T51 cells stored at 25 °C was the differences in colony size at time of enumeration. In addition to colonies normal in size for the assay, a number of smaller colonies were found, indicating a phenotype with a prolonged lag phase. Due to the overall low cell viability, MCPA degradation was not investigated for these cells.

The formulations represented different dehydration methods (*i.e*. freeze drying and fluidised bed drying) but also the formulation excipient/carrier used. The Ficoll formulation could not be used as a carbon source by T51 cells. The second freeze-dried formulation was based on sucrose, which could be used as a carbon source but the formulation contained no other elements needed for growth. However, cottonseed flour is a complex material containing

carbon sources and other growth factors, for instance both arginine and methionine. The effect of an additional carbon source has been reported to either enhance the biodegradation rates of pollutants, by providing carbon and energy for supplementary growth of the degrading bacteria (Cheyns *et al.*, 2012; Hess *et al.*, 1990), or to repress the degradation through competitive inhibition (Lovanh *et al.*, 2002; Muller *et al.*, 1996). In microcosms, the highest MCPA degradation rate was found using the cottonseed-based formulation (**IV**). This is a promising result, since a formulation that supports growth after application can possibly reduce the required inoculum size and thereby the cost. Moreover, addition of the amino acids L-methionine and L-arginine further improved the degradation of MCPA, with less than 1% of added MCPA remaining after 24 h with the cottonseed flour formulation. A nutrient-based formulation can also be used to overcome nitrogen limitation (Jackson *et al*., 1996), as found for MCPA degradation in the ballast soil (**I**). In addition, a carrier-based inoculum is often more robust, as it offers a protective surface after soil inoculation (*i.e.* a physical barrier between the inoculated cells and the surrounding environment) (van Veen *et al*., 1997). However, the low storage stability of the cottonseed flour formulation at elevated temperatures (**IV**) has to be considered.

6.2 Column studies of MCPA leaching

Since the main goal of the bioprophylactic concept is to reduce pesticide transport, column studies were performed to study leaching of MCPA in sand following application of formulated and stored *Sphingobium* sp. T51 cells (**IV**). Dry formulations stored for six months, both fluidised bed-dried and freeze-dried, were rehydrated and added together with the MCPA on the top of the sand (Figure 12). After 24 h, a heavy rainfall event was simulated and the leachate was analysed for MCPA. Each column was inoculated with an equal number of viable T51 cells, and formulated cells were compared with fresh non-formulated T51 cells and an uninoculated control. For all treatments MCPA was detected in the leachate after approximately one pore volume, indicating that no preferential flow of MCPA occurred. After bacterial application only a total of 6-15% of the initially applied MCPA was leached, while 88% of applied MCPA was leached after 10 h in the control without bacterial application and with MCPA still leaching. The fact that formulated cells performed equally well to fresh cells is promising for the bioprophylactic concept. However, the experiments were carried out under optimal conditions that favoured MCPA degradation (*e.g.* temperature and soil water content).

Figure 12. Equipment used for column studies of MCPA leaching.

6.2.1 Soil water content

To approach potential field conditions, the effect of soil water content on MCPA degradation by strain T51 was studied (**IV**). The soil water content has been found to be the most important factor regulating basal respiration and functional diversity in ballast soil (Cederlund *et al.*, 2012). It also affects degradation rates by regulating the microbial activity as well as the overall availability of substrate to microorganisms. The relationship between mineralisation and degradation of MCPA found in Paper **I** suggests that almost the entire amount of MCPA was dissolved in the soil water phase and available for degradation (*e.g.* the degradation curve shifted from exponentially increasing to decreasing rates when the MCPA was almost completely degraded) (Jensen *et al.*, 2004; Bergström & Stenström, 1998). However, soils of interest for the bioprophylactic concept (*i.e.* soils with coarse texture and weak structure) are easily drained and are consequently often dry. Decreased soil water contents during the pre-incubation for 24 h before leaching started in the column studies resulted in lower MCPA degradation and thus increased leaching (**IV**). However, the lowest moisture content of 7% water-holding capacity, which is realistic in light soils, still resulted in 30% degradation of the applied MCPA within 24 h, probably due to the water, the pesticide and the bacteria all being concentrated at the surface of the soil after application.

Nevertheless, this degradation rate is still too low to be acceptable for the bioprophylactic concept. Therefore, formulations or techniques that provide degraders with sufficient water after application in the field have to be identified.

7 The bioprophylactic concept - present status and future challenges

7.1 Present status

The major goal of the work presented in this thesis was to develop a method that addresses and solves the problems of using pesticides on soils with low potential for degradation and adsorption and thereby a high risk of leaching (**I**). The bioprophylactic concept is proposed to be one possible solution to overcome the risk of pesticide water contamination from such soils (**II**). For the development of the concept, the MCPA-degrader *Sphingobium* sp. T51 was isolated from a soil that was highly contaminated with phenoxy acids (**II**). Soil inoculation with this isolate in microcosms significantly enhanced the degradation of MCPA compared with uninoculated soil, without loss of the herbicidal effect (**II**). However, this enhanced MCPA degradation after inoculation could not be repeated under field conditions, and therefore the need for a formulated degrader was obvious. *Sphingobium* sp. T51 was found to have a good inherent capability to survive dehydration, with high initial cell viabilities after both freeze drying and fluidised bed drying (**III**, **IV**). Storage stability of the formulated *Sphingobium* sp. T51 was related to the formulation excipient/carrier used and the storage conditions, *i.e.* temperature and atmosphere (**III**, **IV**). Formulated and long-term stored *Sphingobium* cells maintained their MCPA degradation efficacy and reduced MCPA leaching as efficiently as freshly cultivated cells when equal amounts of viable cells were used for the bioaugmentation (**IV**).

7.2 Future challenges

Although the work in this thesis shows that the concept *per se* has good potential, there is still a long way to go before its practical implementation in the field. In order to obtain an efficient and reliable method, several more factors in addition to those studied and discussed in this thesis have to be considered. Such factors include the effect of pesticide formulation, controlled cell activity after application, environmental conditions and rehydration of dry formulations.

7.2.1 Pesticide formulations

For the bioprophylactic concept, the requirements on the formulation depend, among other things, on the intended method of application, *i.e*. application method of the pesticide and the pesticide-degrading microorganism. One option could be to mix the microorganisms with the diluted pesticide formulation in the spraying tank. The pesticide and its degrader would then be applied in the same droplet, thus minimising the distance between them and allowing good conditions for degradation. However, in addition to the active ingredient(s), pesticide products also contain formulation compounds to optimise the biocidal activity, improve the properties of the product for storage and provide a product that is safe and convenient to use (Knowles, 2007). Formulated MCPA 750 had a detrimental effect on the survival of *Sphingobium* sp. T51 in realistic spraying tank concentrations, *i.e.* 2 kg MCPA 750 and 50, 200 or 400 L of water ha^{-1} (**II**). Consequently, in practical applications the pesticide and pesticide-degrading organism cannot be mixed in the spraying tank, or if so, the organism must be formulated to be protected from direct contact with the liquid.

7.2.2 Controlled cell activity

It is also important that the degrading microorganisms do not interfere with the intended action of the pesticide. Enhanced degradation of MCPA was found in microcosm studies with plants without losing the herbicidal effect (**II**). However, the set-up used gave the least possible contact between the pesticide and its degrader prior to the start of the experiment. The herbicidal effect was also obtained in the field studies, but strain T51 was perhaps not viable for very long and thus did not interfere with the MCPA action. The microorganisms therefore have to be formulated in such a way that their degrading capacity is activated at a suitable time after their application to ensure that the pesticide has had time to be absorbed into the target organism in sufficient amounts to give its intended effect. In relation to rainfall, this is known as the rainfastness

time, *i.e.* the rain-free period required after application of a pesticide to ensure that its intended effect is not reduced by washing-off. This time varies between 10 minutes and 8 hours for 48 foliar herbicide products, with the majority in the time span 1-2 hours and with 4 hours for MCPA (Swedish Board of Agriculture, 2013).

7.2.3 Environmental conditions

Fluctuating and often harsh environmental conditions are difficult to control and can limit pesticide degradation after application in the field (Lestan & Lamar, 1999). The bioprophylactic concept has to function in occasionally dry soil conditions and further studies are therefore needed to find methods and formulations that solve this problem.

Temperature should be considered already during cultivation of the microorganism, since this can influence survival rates and cell viability after dehydration and subsequent storage (Schoug *et al.*, 2008) and perhaps also activity under unfavourable soil temperatures in the field.

Sphingobium sp. T51 showed a relatively narrow pH range for growth activity of 6.5-7.5, with an optimum at 7.0. This is in agreement with a previous report on a related *Sphingomonas* strain that was highly sensitive to minor changes in soil pH (Bending *et al.*, 2003). Studies have shown that pesticide degradation rate in topsoil can be highly variable within the field, with pH controlling the dynamics of organisms responsible for growth-linked pesticide degradation (Walker *et al.*, 2001). Hence, soil pH may limit the use in field applications if not carefully considered.

UV radiation is an important factor affecting microorganisms after release in the field. Sensitivity to UV radiation is one of the main limitations in the commercial development of biocontrol products (Cohen & Joseph, 2009; Ragaei, 1999) and possibly also for the bioprophylactic concept.

7.2.4 Rehydration of dry formulations

It could be argued that dry conditions are not a problem, since leaching is often low under such conditions. One possible method for bacteria inoculation could thus be in dry form, without rehydration of the bacteria prior to application. The degrader could then rehydrate and be metabolically activated at the time of a rainfall event, which would coincide with the risk of increased pesticide leaching. However, even if the bacteria were to survive until a rainfall, they could nevertheless still lose their viability during rehydration. Cell survival during rehydration depends among other factors on the rehydration medium (Costa *et al*., 2000), the rehydration temperature (Ray *et al*., 1971) and the rate

of dehydration (Poirier et al., 1999). Rehydration in the field is therefore difficult to control. In addition, this approach may not be suitable due to the high cell viability losses of the dried material obtained in the presence of air (**III**, **IV**). Moreover, considering the decrease in pesticide bioavailability with time, fast degradation is always preferable.

7.2.5 Could bioencapsulation be a solution?

Different encapsulation techniques have been proposed to overcome many of the above-mentioned problems, including delayed activation of the microorganisms and protection against severe environmental factors (Anal & Singh, 2007; Bjerketorp *et al.*, 2006; Cokmus & Elcin, 1995; Elcin *et al.*, 1995). In addition, encapsulation can increase storage stability (Bjerketorp *et al.*, 2006). In the present context, bioencapsulation is the embedding of microorganisms in a semipermeable material, alginate being the most frequently used (Somtrakoon *et al.*, 2009; Zohar-Perez *et al.*, 2003; Selmer-Olsen *et al.*, 1999; Smidsrød & Skjåkbræk, 1990). Alginate encapsulation of strain T51 has been successfully performed, with maintained MCPA degradation activity after release (L. Pizzul, pers. comm. 2013). However, common methods used for alginate encapsulation (Smidsrød & Skjåkbræk, 1990) have some limitations, such as scale-up restrictions and large beads (Sohail *et al.*, 2011), that are unfavourable for the bioprophylactic concept. In addition, it would be preferable to encapsulate cells in a material not permeable to molecules larger than small molecules such as oxygen and water, in order possibly also to eliminate the problem of harmful compounds in the pesticide formulation.

The CaptiGel encapsulation technique, using a titanium dioxide-based sol-gel material (Kessler *et al.*, 2008), was studied for strain T51. The biocompatibility of this gel has been tested previously by encapsulation of a number of model microorganisms, *e.g.* the chlorophenol degrader *Arthrobacter chlorophenolicus,* the yeast *Pichia anomala* and the lactic acid bacterium *Lactobacillus plantarum* (Kessler *et al.*, 2008). The yeast and *L. plantarum* were successfully encapsulated and released with high survival rates, while the survival of the *Arthrobacter* strain was noticeably lower. CaptiGel encapsulation of strain T51, both with active bacteria in culture and cells dried prior to encapsulation, resulted in low initial cell survival, typically around 1-5%. It is not known whether the cells lose viability during the encapsulation process itself, or whether the release is the critical step, for instance due to the pH of the buffer used for dissolving the gel. Use of microbeads produced by an aerosol technique that combines alginate encapsulation with a chitosan coating has been successfully demonstrated in protection of a *Lactobacillus* strain in

the bile environment (Sohail *et al.*, 2011). Thus, a combination of alginate encapsulation with a coating shell may be a possible approach for formulation of bacterial strains to be used for the bioprophylactic concept or similar applications. Preliminary results using a combination of the two encapsulation techniques, *i.e.* encapsulation of the cells in alginate and thereafter coating the beads with CaptiGel, show increased survival rates to 20-30% of *Sphingobium* sp. T51 (L. Pizzul, pers. comm. 2013).

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